Improved bioassay method for *Spodoptera litura* chitinase inhibitors using a colloidal chitin powder with a uniform particle size as substrate

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Abstract: A previously reported bioassay method for *Spodoptera litura* chitinase inhibitors has been improved by use of colloidal chitin powder with a uniform particle size. This improvement made the assay four times more sensitive. Detection of three active supernatants by screening of supernatants and cell extracts from 135 fermentation broths has proved the efficiency of this improved method.

Keywords: chitinase inhibitory assay; colloidal chitin; *Spodoptera litura*

1 INTRODUCTION

Chitinase, a key enzyme in the moulting of insects, is expected to be a target of biorational insect growth regulators. Since the only insect chitinase inhibitors reported so far are allosamidin and its derivatives,[1–5] a search for novel insect chitinase inhibitors by a more efficient and simple bioassay is desirable. The previously reported method[6] using a chitinase solution simply prepared from *Spodoptera litura* (F) pupae had one serious disadvantage, that colloidal chitin prepared by the method of Jeuniaux[7] was not always dispersed homogenously enough to act as a good substrate for the assay. Since the biodegradation of colloidal chitin is monitored by a decrease in A_{610} in the assay, homogenous dispersion of colloidal chitin is important for the assay. Therefore, the previously reported method was sometimes poor in both sensitivity and reliability. The present paper describes the preparation of a colloidal chitin powder with a uniform particle size and its use as a substrate for the chitinase reaction. The efficiency of the new method, using the improved substrate, was confirmed by a well-known inhibitor, allosamidin and tested by screening microbial cultures for chitinase inhibitors.

2 MATERIALS AND METHODS

2.1 General

Allosamidin was a gift from Dr S Sakuda (University of Tokyo). Chitin powder was purchased from Wako Pure Chemical Industries (Osaka, Japan). Chitinase solution from *S litura* pupae was prepared by the method of Kawazu *et al*.[6]

2.2 Preparation of colloidal chitin

Colloidal chitin was prepared from chitin powder by a modification of the method of Shimahara and Takiguchi.[8] Chitin powder (2.5 g) was added slowly into concentrated hydrochloric acid (100 ml) below 5 °C with stirring. After 10 min, the suspension was heated gently up to 37 °C and kept for 30 min with stirring. The suspension became clearer during the stirring. The suspension was filtered through a glass filter (25GP100, Sibata Scientific Technology, Tokyo, Japan). The filtrate was poured into one litre of deionized water below 5 °C with vigorous stirring. Within a few minutes, the solution became turbid because of the reprecipitation of chitin. After 30 min, stirring was stopped, and the suspension was kept overnight below 5 °C. The precipitate was washed with deionized water by decantation until the pH rose above 5, and filtered through the above glass filter to remove particles larger than 100 μm in diameter. Chitin particles ranging from 10 to 100 μm in diameter were collected by filtration with a glass filter (17GP16, Sibata Scientific Technology) and washed with deionized water until the washings became neutral. These particles were washed with methanol and diethyl ether and dried to a fine powder (1.6 g). The powder was dispersed into deionized water by ultrasonication (Branson B-12, Branson Ultrasonics Corp, Danbury, USA) just before use as a substrate.

2.3 Chitinase assay

The chitinase activity was determined by the method of Kawazu *et al*[6] with some modifications. The reaction mixture was composed of 20 μl of the colloidal

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chitin suspension and 380 µl of the enzyme solution in a 52.6 mM citrate-phosphate-borate buffer (pH 7.0). The generated *N*-acetylglucosamine was determined by a modification[9] of the method of Morgan and Elson. One unit of the chitinase activity was defined as the amount of the enzyme that yielded 1.0 µmol of *N*-acetylglucosamine per minute.

2.4 Assay method of chitinase inhibitory activity

Inhibitory activity against chitinase from *S litura* was determined by the method of Kawazu *et al*.[6] with some modifications. The test mixture contained 20 µl of the colloidal chitin suspension, 180 µl of the enzyme solution in a 111 mM citrate-phosphate-borate buffer (pH 7.0), and 200 µl of an aqueous solution of test material. If the test material was insoluble in water, it was dissolved in methanol (less than 10 µl) and the same volume of methanol was added to the control.

2.5 Fermentation of micro-organisms

Twenty-seven strains of soil microorganism were cultured at 28 °C for five days on a reciprocal shaker (2 cm, 320 strokes min^{-1}) in 10 ml of five different media, PSM, Bennett's, CM, H, and N (see below). The culture broth was filtered through cotton to separate the cells from the supernatant. An aliquot (200 µl) of the supernatant was tested for chitinase inhibitory activity. The cells were soaked in acetone+methanol (1+1 by volume) at room temperature for three to four days, and the extract was concentrated to dryness. The dried extract was suspended in 25 µl of methanol. To the suspension, 475 µl of water was added with stirring. A portion (200 µl) of the suspension was tested for chitinase inhibitory activity.

2.6 Media for fermentation

PSM medium (pH 5.5–6.5): peeled potato 300, sucrose 20.0, malt extract (DIFCO, Detroit, USA) 2.0 (g $litre^{-1}$ in water).

Bennett's medium (pH 7.3): glucose 10.0, yeast extract (Nacarai Tesque, Kyoto, Japan) 1.0, bonito extract (Wako Pure Chemical Industries) 1.0, NZ Amine Type A (Humko Sheffield Chemical, Norwich, USA) 2.0 (g $litre^{-1}$ in water).

CM medium (pH 7.0): glucose 10.0, glycerol 5.0, corn steep liquor (Ajinomoto, Tokyo, Japan) 3.0, beef extract (DIFCO) 3.0, yeast extract 3.0, calcium carbonate 2.0, thiamine 0.01 (g $litre^{-1}$ in water).

H medium (pH 7.2): glucose 15.0, glycerol 10.0, soy bean meal (Nisshin Oil Mills, Tokyo, Japan) 15.0, dry yeast (Asahi Breweries, Tokyo, Japan) 5.0, sodium chloride 5.0, ammonium sulfate 5.0, corn starch 10.0, Polypepton (Nihon Pharmaceutical, Tokyo, Japan) 10.0, cane molasses (Ajinomoto) 20.0, beef extract 10.0, calcium carbonate 4.0 (g $litre^{-1}$ in water).

N medium (pH 6.4): soybean meal 15.0, ammonium sulfate 2.0, dry yeast 2.0, starch 25.0, sodium chloride 5.0, calcium carbonate 4.0 (g $litre^{-1}$ in water).

3 RESULTS AND DISCUSSION

3.1 Establishment of conditions for the sensitive chitinase inhibitory assay

For a sensitive and reliable chitinase inhibitory assay, the particle size of colloidal chitin powder needed to be small and uniform. Hydrochloric acid treatment of chitin and subsequent selection of particles of uniform size by filtration gave satisfactory colloidal chitin powder. This colloidal chitin powder could be dispersed into water more homogeneously than colloidal chitin powder used in the previous method.

The optimal composition of the assay mixture including newly prepared colloidal chitin was re-examined and fixed as follows: 20 µl of 6 mg ml^{-1} colloidal chitin suspension, 180 µl of the enzyme solution (8.3×10^{-3} units) in 111 mM citrate-phosphate-borate buffer (pH7.0), and 200 µl of an aqueous solution of test material. A linear relation was obtained between the decrease in A_{610} and the amount of *N*-acetylglucosamine generated from the colloidal chitin by *S litura* chitinase. Generation of 64 nmol of *N*-acetylglucosamine corresponded to a decrease in A_{610} by 0.1 in this improved method, but to a decrease by 0.02 in the previous method. This means that this improved method could be five times more sensitive. Elevation of the sensitivity in the improved method can be accounted for by chitin particles of smaller size (10 ~ 100 µm) than before (≧100 µm). The decrease in turbidity of the reaction mixture following the degradation of colloidal chitin is assumed to be faster, in part due to a decrease in particle size of chitin. The uniformity of the particle size of colloidal chitin also may considerably contribute to the sensitivity and reliability of the assay.

3.2 IC_{50} of allosamidin in the improved assay method

The IC_{50} values of allosamidin in this improved assay method (Fig 1), the previous assay method,[6] and the *Bombyx mori* L chitinase inhibitory assay[3] using γ-chitin red as a substrate were 50, 200, and 700 nM, respectively. These values confirm that this assay is

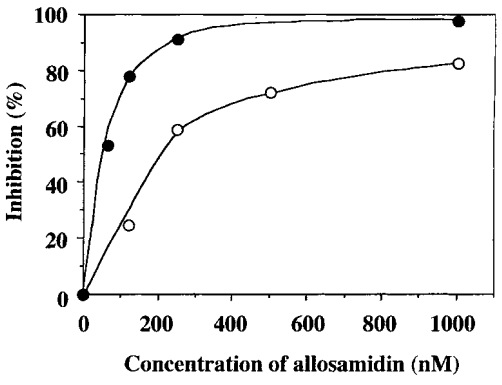

Figure 1. Inhibitory activity of allosamidin against *Spodoptera litura* chitinase in the (○) previous and (●) present assay methods.

Table 1. Inhibitory activity of the supernatants from microbial cultures against *Spodoptera litura* chitinase

Origin of supernatant	ID_{50} (μl)
A1 strain in Bennett's medium	28
A13 strain in N medium	46
A13 strain in Bennett's medium	48

four times more sensitive than the previous assay and 14 times more sensitive than the *B mori* chitinase inhibitory assay.

3.3 Screening of microbial cultures for insect chitinase inhibitors

Screening of the supernatants and cell extracts from 135 fermentation broths using this improved assay revealed three active supernatants, strain A1 in Bennett's medium and strain A13 in Bennett's and N media, which showed more than 70% inhibitory activity at a dose of 200 μl of the culture supernatant. The 50% inhibitory dose (ID_{50}) values for these active supernatants are listed in Table 1. These active supernatants were not detected by our previous assay because of its lower sensitivity to chitinase inhibitors. These results indicate that this improved assay with uniform and small-sized chitin particles is the most sensitive assay for insect chitinase inhibitors and a useful means for more efficient search for novel chitinase inhibitors.

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